IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	AKIYAMA et al.	Examiner:	Qian Janice Li
Application No.:	10/594,695	Group Art Unit:	1633
Filed:	September 28, 2006	Confirmation No.:	8066
Docket No.:	3190-101	Customer No.:	33432

For: sFRP EXPRESSION ENHANCING AGENT

DECLARATION UNDER 37 C.F.R. §1.132

1) I, Tetsu Akiyama, am one of the named inventors in the above-identified U.S. patent application.

2) I am familiar with the present application and the Office Action dated August 4, 2009.

3) The experiments described herein were conducted under my direction and/or supervision.

4) As is shown from the experiments described below, the identification method of the present invention can identify a compound having an effect of enhancing expression and/or function of sFRP.

5) Experiments were conducted to determine whether over-expression of Runx in Dlg -/- MEF (embryonic fibroblast) can induce change of sFRP expression, using RT-PCR analysis. The effect of co-over-expression of Runx with Dlg on sFRP expression was also investigated. A Runx family is known to include three members, Runx1, Runx2 and Runx3. It has been reported that Runx1 and Runx3 are expressed and function in blood cell lineage. Therefore, we focused on these two Runx members and used them in the experiments described

below. Transfected cells were enriched by Auto-MACS, because of low transfection efficiency into MEF. The experiments were conducted as follows:

a. Gene transfection into MEF and Enrichment of transfected cells by Auto-MACS

Mouse cell lines, #33 *Dlg* +/+ MEF and #33 *Dlg* -/- MEF, were generated from a Wild-type and Dlg-/- mouse, respectively, and used in the experiments. Gene transfection into MEFs was carried out by lipofection using Lipofectamine/PLUS reagent (Invitrogen). MEFs were plated in 10 cm dishes, and then plasmids described in Table 1 were added at the dose indicated to each of the dishes to co-transfect the plasmids into the MEFs according to the manufacturer's protocol.

Table 1

Mouse cell line	Plasmid	Amount of Plasmid	Number of dishes
Dlg +/+ MEF	pCAGGS	1.9 μg	×10
	pMkitneo	1.9 μg	
	pMACS-K^kII	0.2 μg	
Dlg -/- MEF	pCAGGS	1.9 μg	×10
	pMkitneo	1.9 μg	
	pMACS-K^kII	0.2 μg	
	pCAGGS-HA-mRunx1/3	1.9 μg	×10
	pMkitneo	1.9 μg	
	pMACS-K^kII	0.2 μg	
	pCAGGS-HA-mRunx1/3	1.9 μg	×10
	pMkitneo-hDlg	1.9 μg	
	pMACS-K^kII	0.2 μg	

Ten dishes were prepared for each MEF in order to obtain a large amount of cells. The transfected cells were then sorted by Auto-MACS (Daiichi Pure Chemicals Co., Ltd.). The plasmid pMACS Kk.II expresses a H-2K^k surface marker that lacks an intracellular domain. Twenty four hours after the transfection, the transfected cells were treated with 0.25% tripsin/PBS-EDTA [0.25% Trypsin (1:250) (Gibco), 0.02% EDTA (ethylenediaminetetraacetic acid), PBS (phosphate buffered saline)] for a short time period to detach the cells from the dishes and were quickly added with 6.5 ml of PBS containing 5% FBS (fetal bovine serum) in each 10 cm dish to stop the trypsin. The transfected cells were washed once with PBS. The transfected cells collected from five dishes were resuspended in 2ml of PBS containing 5% FBS, followed by addition of 80 µl of MACSelect K^k microbeads (Miltenyi Biotec), and incubated at room temperature for 15 minutes. After centrifugation of the resultant at 1,000 rpm for 3 minutes and discarding the supernatant, the pellet was resuspended in PBE [PBS, 0.05% bovine serum albumin (BSA) (Nacalai), 5 mM EDTA] and sorted by Auto-MACS using POSSEL_S sorting program.

b. SDS-PAGE and Western Blotting

The transfected cells were dissolved with 1% Triton X-100 lysis buffer [1% Triton X-100 (SIGMA), 50 mM Tris–HCl pH 7.5, 150 mM NaCl, 5 mM EDTA, 1 mM PMSF (phenylmethylsulfonyl fluoride), 5 µg/ml aprotinin, 5 µg/ml leupeptin]. After mixing on a rotator at 4°C for 30 minutes, centrifugation was carried out at 15,000 rpm for 20 minutes to collect the supernatant. The thus collected cell lysate solution was added with 6× sample buffer [350mM Tris-HCl pH6.8, 10% SDS (sodium dodecylsulphate), 36% glycerol, 0.93% DTT (dithiothreitol), 0.012% BPB (bromphenol blue)] and denatured at 94°C for 3 minutes. Proteins were separated by SDS-PAGE in 8% acrylamide gel. The gel was then equilibrated in a transfer buffer

[0.0374% SDS, 24 mM Tris, 192 mM glycine, 20% methanol], and transferred onto Immunobilon-P membrane (Millipore) by a semi-dry system. The membrane was blocked with 5% skim milk/TBST [0.2% Tween20, 50 mM Tris–HCl pH 7.5, 150 mM NaCl] for 30 minutes, and then reacted with a primary antibody diluted with TBST at 4°C overnight. Both an anti-Dlg monoclonal antibody (BD Transduction Lab.) and an anti-HA monoclonal antibody (3F10: Roche) were diluted at 1:1,000 for use. AP(alkaline phosphatase)-conjugated mouse anti-rabbit IgG antibodies (Promega) were diluted at 1: 2.000 for use as a secondary antibody. After eight hours reaction, the membrane was subjected to AP substrate buffer [100 mM Tri-HCl pH9.5, 100 mM NaCl, 50 mM $MgCl_2$] containing NBT/BCIP (Promega) for color development, and then dipped in MiliQ at room temperature for an appropriate time period to detect the target protein.

c. RT-PCR

Total RNA was collected using ISOGEN (NIPPON GENE) according to the manufacturer's protocol. 10 μg of total RNA was used for reverse transcriptase reaction using SuperScript III reverse transcriptase (Invitrogen). The reaction condition was similar to that shown in the manufacturer's protocol.

Primer sequences used in the PCR are shown below.

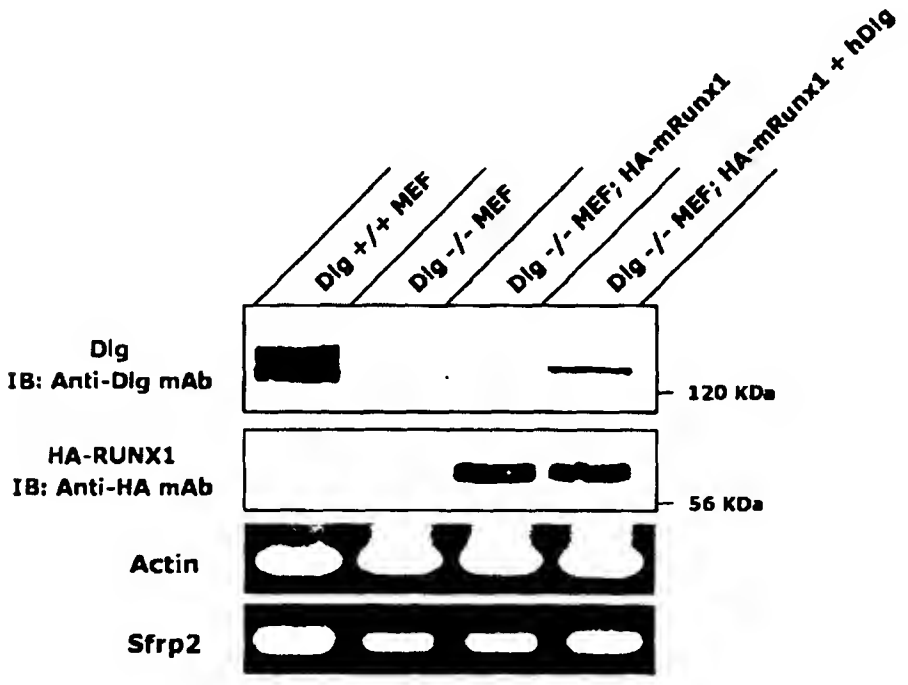
Mouse	forward	5') GGGGTCACCCACACTGTGCCCATCTACGAG (3'
Actin	Reverse	5') ACTCCTGCTTGCTGATCCACATCTGCTGGA (3'
Mouse	forward	5') TCTTCGGCCAGCCCGACTTCTCCTACAAGC (3'
Sfrp2	Reverse	5') CTAGCATTGCAGCTTGCGGATGCTGCGGGA (3'

The PCR was initiated as Hot Start PCR at 94°C for 2 minutes followed by denaturation at 98°C for 10 seconds, annealing at 55°C for 30 seconds, and extension at 72 °C for 1 minute.

Cycle conditions were 26 cycles and 30cycles for Actin and sFRP2, respectively. Reagents used in the PCR, such as Taq polymerase and buffers, are those contained in Ex-Taq (TaKaRa).

6) According to the results obtained from the experiments described above, Runx1 and Runx3 both showed an effect of increasing sFRP2 expression in a cell lacking both Dlg alleles, which was further increased by co-expression with Dlg (Figs. A and B, respectively). These results suggest that a compound having an effect of enhancing expression and/or function of Runx can enhance sFRP2 expression in a cell lacking both Dlg alleles, and such a compound should be one example provided with the method of the present invention.

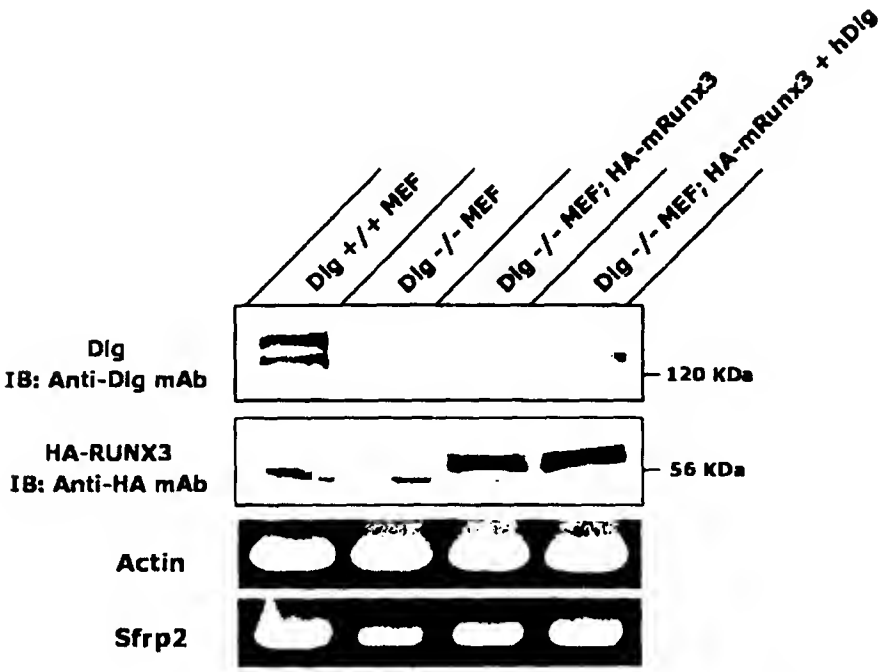
(A)



The expression of mRunx1 and hDlg was analyzed by Western Blotting, and the expression of sFRP was analyzed by RT-PCR.

sFRP2 expression was increased by forced expression of mRunx1, which was further increased by forced expression of hDlg together with mRunx1.

(B)



The expression of mRunx3 and hDlg was analyzed by Western Blotting, and the expression of sFRP was analyzed by RT-PCR.

sFRP2 expression was increased also by forced expression of mRunx3, which was further increased by forced expression of hDlg together with mRunx3.

7) I declare further that all statements made herein of our own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code and any such willful false statement may jeopardize the validity of the

application or any issuing thereon.

Date: January 13th. 2010

TETSU AKIYAMA